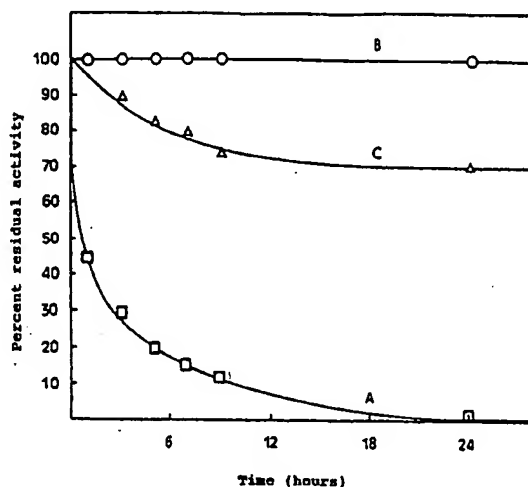




## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(21) International Application Number: PCT/EP93/00205 (22) International Filing Date: 29 January 1993 (29.01.93) (30) Priority data: MI92A000162 29 January 1992 (29.01.92) IT (71) Applicant (for all designated States except US): CONSIGLIO NAZIONALE DELLE RICERCHE [IT/IT]; Piazzale Aldo Moro, 7, I-00185 Roma (IT). (72) Inventors; and (75) Inventors/Applicants (for US only): VERONESE, Francesco, Maria [IT/IT]; CALICETI, Paolo Dipartimento di Scienze Farmaceutiche [IT/IT]; (C.di Chimica del Farmaco e dei Prodotti Biologicamente Attivi del CNR), Università di Padova, Via F. Mazzolo, 5, I-35131 Padova (IT). SCHIAVON, Oddone Dipartimento di Scienze Farmaceutiche [IT/IT]; (C.di Chimica del Farmaco e dei Prodotti Biologicamente Attivi del CNR), Università di Padova, Via F. Mazzolo, 5, I-35131 Padova (IT). SARTORE, Luciana Dipartimento di Scienze Farmaceutiche [IT/IT]; (C.di Chimica del Farmaco e dei Prodotti Biologicamente Attivi del CNR), Università di Padova, Via F. Mazzolo, 5, I-35131 Padova (IT).		(74) Agent: MINOJA, Fabrizio; Studio Consulenza Brevettuale, Via Rossini, 8, I-20122 Milano (IT). (81) Designated States: AU, BB, BG, BR, CA, CZ, FI, HU, JP, KP, KR, LK, MG, MN, MW, NO, NZ, PL, RO, RU, SD, SK, UA, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG). Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: A METHOD TO MAINTAIN THE ACTIVITY IN POLYETHYLENE GLYCOL-MODIFIED PROTEOLYTIC ENZYMES



Autolysis of native trypsin ( $\square$ ), A; PEG-trypsin randomly modified with no site-protection ( $\circ$ ), B; PEG-trypsin modified in heterogeneous phase in the presence of benzamidine-Sepharose ( $\triangle$ ), C.

## (57) Abstract

A method to prevent the loss of enzymatic activity of proteolytic enzymes toward macromolecular substrates, loss occurring when said enzymes are modified with polyethylene glycol to increase the applicative potentials thereof in the biomedical field or in biocatalysis.

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A METHOD TO MAINTAIN THE ACTIVITY IN POLYETHYLENE  
GLYCOL-MODIFIED PROTEOLYTIC ENZYMES

The present invention relates to a method to avoid binding of polyethylene glycol (PEG) at the active site and its surroundings in proteolytic enzymes.

5 The method, based on the modification reaction of an enzyme-macromolecularized inhibitor complex in the heterogeneous state, allows to obtain enzyme-PEG adducts in which the proteolytic activity toward macromolecular substrates is preserved.

**BACKGROUND OF THE INVENTION**

10 The modification of enzymes with polyethylene glycol (PEG) is a technology that has markedly been developed in recent years to obtain adducts having valuable properties for the use both in the biomedical field and as novel biocatalysts, due to the presence of  
15 polyethylene glycol chains linked at the surface.

In fact, the enzyme-PEG adducts lose the major part of the typical properties of naturally occurring enzymes, such as immunogenicity and antigenicity, rapid clearance from circulation, easy degradability by  
20 proteases and instability in diluted solutions [A. Abuchowski et al., J. B. C., 252 3582, 1977], that often prevent their use in therapy.

In the use of enzymes in biocatalysis, the PEG-enzyme adduct acquires a quite different  
25 characteristic, i.e. the solubility in organic solvents, thus allowing a better use of the enzymes in converting liposoluble substrates [Y. Inada et al., Trends Biotech., 4 190, 1986].

The properties of such novel biotechnologic products are due to the fact that PEG binds to the enzyme surface, thus protruding with its hydration cloud toward the outer protein solvent, preventing the access of large molecules, such as proteolytic enzymes, as well as the recognition by the immune system. On the other hand, as PEG also has amphiphilic properties, the PEG-enzyme adduct can acquire solubility in organic solvents.

10 However, the polymeric cloud surrounding the PEG-enzyme adduct also limits the general use of said derivatives: in fact, the enzymatic activity is maintained toward small substrates, that can have access to the active site diffusing among the PEG  
15 polymer chains, but it is prevented toward large substrates, that cannot reach the active site due to steric hindrance.

Therefore, convenient PEG-enzyme adducts are obtained with enzymes such as superoxide dismutase, catalase, asparaginase, arginase, urease, adenosine  
20 deaminase, phenylalanine ammonium liase etc., which are nowadays under pharmacological and clinical tests, but not with enzymes acting on large substrates such as proteins, nucleic acids and polysaccharides. In fact, substantial activity losses are described following a  
25 PEG-modification of trypsin, chymotrypsin, urokinase, ribonuclease, lysozyme and the like.

A proposed solution consists in preparing adducts having only a few polymer chains linked to the enzyme, thus decreasing the loss in enzymatic activity.  
30 However, this result, which can be attained carrying

out the reaction in a PEG molar defect, suffers from drawbacks such as attainment of very heterogeneous products and poor reproducibility.

#### SUMMARY OF THE INVENTION

5 To allow access of macromolecular substrates, large polypeptides or proteins, in case of proteolytic enzymes, to the active site, the PEG binding to the enzyme is carried out in heterogeneous phase, in which the enzyme is linked to an inhibitor thereof that is,  
10 in its turn, immobilized on a highly hydrated insoluble polysaccharide (Sephacrose). In such a way, the PEG polymer will bind to enzyme areas far from the active site and its proximity, thus allowing the approach of the substrate macromolecules.

15 The method was investigated with two serine-dependent proteolytic enzymes, trypsin and urokinase, the first being used in medicine, for instance in the removal of necrotic tissues, in digestive disorders or in ophthalmology in the elimination of protein deposits  
20 from contact lenses; the latter, i.e. urokinase, being of specific therapeutical interest as a plasminogen activator.

Benzamidine, an inhibitor of serine enzymes, was used as a linker to keep Sepharose in the surrounding  
25 of the active site (example 2). The method could also be used for the site-protection of other serine enzymes, such as tissue plasminogen activator (tPA), plasmin, chymotrypsin, elastase, kallikrein and the like.

30 The following examples report the comparison of the activities of the starting enzyme A), PEG-modified

either with no protection, or in the presence of free benzamidine in solution B) and obtained carrying out the modification in heterogeneous phase with the complexed enzyme with the benzamidine-Sepharose macromolecularized inhibitor C) (examples 1, 3, 5, 7).

The obtained results will be described with reference to the figures and tables reported in the following.

In the various modifications, monomethoxypolyethylene glycol of MW 5000 (PEG) was used, bearing norleucine as a spacer between polymer and protein, activated at the carboxy group as the succinimidyl ester. PEG with norleucine was used as it allows a precise evaluation of the linked polymer chains, by means of amino acids analysis [L. Sartore et al., Appl. Biochem. Biotechnol., 27 45, 1991]. Trypsin and urokinase used in the tests were previously purified by affinity chromatography. In the reported examples, the activities of the enzyme various forms (A, B, C) were compared for equimolecular amounts of enzyme, i.e. without taking into account the weight of the bond polymer.

Table 1 reports the esterase activity toward the small substrate tosyl-arginyl-methyl ester (TAME) of: 1) native trypsin A), trypsin randomly modified with PEG B) and trypsin linked to benzamidine-Sepharose C). In these cases, the catalytic activity is not lost, on the contrary, a small increase in activity occurs in the PEG-modified adducts, which proves that PEG chains do not prevent the access to the active site of a small substrate (example 4). Similar increases in activity

have already been observed in proteolytic enzymes, following different chemical modifications.

The same Table also reports the hydrolytic activity toward casein 2a), that is a protein of MW 23,600 turning out to be strongly lowered to about 30%, following a random modification with PEG B), whereas it is totally maintained when the modification is carried out in heterogeneous phase with trypsin linked to benzamidine-Sepharose C) (example 5).

Finally, Table 1 shows that the hydrolytic activity toward bovine serum albumin (BSA) 2b), that is a protein of higher molecular weight, namely 64000, is completely lost in the sample that was PEG-modified in the absence of site-protectors B), whereas a high activity degree is still retained (55% compared with native trypsin) when the modification is performed with trypsin linked to benzamidine-Sepharose C) (example 5).

Figure 1 shows the autolysis of the various trypsin sample, evaluated by means of the determination of the esterase activity. Trypsin in aqueous solution undergoes degradation very rapidly; when it is randomly modified with PEG B) it does not undergoes self-digestion, whereas the one obtained carrying out the modification in the presence of benzamidine-Sepharose C) shows a slow starting loss of activity, that however becomes stable at about 70%. This behaviour is in agreement with the above results, namely the incapability of randomly modified trypsin B) to digest a large molecule (in the case represented by trypsin itself). The decreased autolysis of the sample modified as a benzamidine-Sepharose complex C) is consistent, on

the one hand, with the persistence of the proteolytic activity of this species toward macromolecular substrates, on the other with a more difficult access for proteases, due to steric hindrance of the PEG chains masking the enzyme surface, with the exception of the active site (example 6).

Table 2 reports the properties of native and modified urokinases, measured by means of the esterolytic activity on a small substratum, namely carbobenzoxy lysine-p-nitrophenyl ester 1), the thrombolytic activity on the synthetic thrombus by means of the resistance to penetration of a glass bead 2) and the affinity to the synthetic thrombus by means of colorimetric measurements of thrombus degradation products 3), eventually by the evaluation of the capability to hydrolyse free plasminogen in solution 4).

In this case also samples of native urokinase A), randomly PEG-modified urokinase B) and urokinase modified by site-protection linking urokinase to benzamidine-Sepharose C) were compared.

Table 2 shows that, analogously to trypsin, the activity toward a small substrate increases (example 9).

On the contrary, thrombolytic activity on the synthetic thrombus, measured by means of the resistance to the penetration of a glass bead, disappears in the randomly modified urokinase sample, whereas the samples obtained by site-protection keep a high thrombolytic activity (example 10).

The affinity to a synthetic thrombus decreases of



10 orders of magnitude for sample C (modified in the presence of benzamidine-Sepharose, and of about 500 orders of magnitude for sample B) (obtained by random modification) (example 11).

5       The activity toward plasminogen in solution decreases by one order of magnitude in sample C), and by 2 orders in the randomly modified sample B) (example 12).

10       Example 1) Preparation of trypsin-PEG in conditions of random modification

10 mg di trypsin are dissolved in 2 ml di borate buffer 0.2 M, pH 8.0 and added under stirring to 160 mg of activated PEG, to obtain a molar ratio of the amino groups present in the protein to PEG of 1:5, while pH is kept to 8.0 in a pHstat with 0.2 M NaOH. After 30' the solution is diluted with 8 ml of HCl 10 mM and it is ultrafiltered with a membrane of cut-off 10,000 to reduce the volume to 2 ml. The dilution and concentration process is repeated for 5 times. Finally, 20 the solution is purified by gel filtration chromatography and the trypsin-PEG peak is concentrated by ultrafiltration to 2 ml, added again with 8 ml of 10 mM HCl and ultrafiltered repeating the procedure for 5 times. (The modification was carried out also in the 25 presence of benzamidine for an enzyme/benzamidine 1:100 molar ratio and the obtained sample was purified as reported above).

The trypsin-PEG adduct has about 13 PEG chains linked per enzyme molecule.

30       Example 2) Preparation of benzamidine-Sepharose

      Sepharose 6B (50 ml) activated by CNBr [P.

Cuartecadas, J. B. C., 245 3059, 1970] is reacted in borate buffer 0.1 M pH 9.5 with 200 mg of p-aminobenzamidine dissolved in 25% dimethylformamide 25%. The resin was washed with 25% dimethylformamide in borate buffer and finally with 0.5 M NaCl, in which it was subsequently preserved. The resin has 5-20  $\mu$ M of benzamidine per ml. A commercially available benzamidine-Sepharose resin can also be used.

10 Example 3) Preparation of trypsin-PEG in conditions of protection of the active site and the surroundings thereof

15 15 mg of trypsin are dissolved in 5 ml of 0.2 M borate buffer , pH 8.0 and added to 5 ml of benzamidine-Sepharose resin, previously washed with 100 ml of 1.5 M NaCl and equilibrated with 0.2 M borate buffer, pH 8.0. After addition of trypsin, the resin is filtered, washed 3 times with 10 ml of 0.2 M borate buffer, pH 8.0 and further filtered.

20 The resin is added with 5 ml of borate buffer and, under stirring, with 360 mg of activated PEG, to reach a 1:7.5 protein amino groups to PEG molar ratio. The suspension is stirred for 1 hour, filtered and washed 3 times with 10 ml of borate buffer. The trypsin-PEG adduct is removed from the resin by repeated washings  
25 (10 times) with 10 ml of 10 mM HCl. The solution is concentrated by ultrafiltration on a membrane and purified by gel filtration chromatography, as in example 1. The trypsin-PEG peak is collected and finally concentrated to 2 ml and diluted with 8 ml of  
30 10mM HCl and concentrated to 2 ml. This procedure is repeated for 5 times. The modified protein contains

about 12 polymer chains per enzyme molecule.

Example 4) Enzymatic activity of trypsin and PEG-trypsin toward a low molecular weight substrate

5 This activity is measured using N $\alpha$ -p-tosyl-arginyl-methyl ester (TAME) as the substrate. The increase in optical density of a solution of 800  $\mu$ l of 0.046 M Tris HCl, 0.015 M CaCl<sub>2</sub> at pH 8.0, 100  $\mu$ l of 1 mM HCl, 100  $\mu$ l of a 0.01 M substrate aqueous solution and 0.5 to 6  $\mu$ g of enzyme.

10 The activity of the modified products is expressed as a percentage compared to the activity of the native enzyme (Table 1).

Example 5) Activity of trypsin and PEG-trypsin toward a high molecular weight substrate

15 To evaluate the protease activity toward high molecular substrates, casein (MW 23000) or serum albumin (MW 64000) were used as standard substrates. A solution of 0.4 ml of 0.1 M Tris HCl, pH 8.0, 0.4 ml of a 1% substrate solution in 0.1 M Tris HCl, pH 8.0 and  
20 an enzyme amount from 0.25 to 3  $\mu$ g is incubated at 30°C for 20 minutes. This solution is added with 1.2 ml of 5% trichloroacetic acid and the optical density at 280 nm is evaluated on the supernatant after centrifugation and removing of the precipitate. The residual activity  
25 of the PEG-trypsin products is evaluated as a percent activity of the native form (Table 1).

Example 6) PEG autolysis of trypsin and its derivatives

0.25 mg of enzyme or PEG-trypsin samples in 1 ml of 0.1 M Tris buffer at pH 8.0 are incubated at 37°C.  
30 The esterase activity test is carried out at preset times to evaluate the percentage of still active enzyme

(fig.1).

Example 7) Preparation of PEG-urokinase in random modification conditions

2.5 mg of urokinase in 2 ml of 0.2 M borate  
5 buffer, pH 8.0, in a polyethylene container, are added  
under stirring with 30 mg of activated PEG at a protein  
amino groups to PEG 1:5 molar ratio. After 60 minutes  
the solution is diluted with 8 ml of 10 mM HCl and  
concentrated by ultrafiltration. The PEG-urokinase  
10 adduct is purified following the procedure of example  
1, always working with polyethylene containers. (The  
modification was carried out also in the presence of  
benzamidine for a 1:100 urokinase/benzamidine molar  
ratio and the obtained sample was purified as reported  
15 above). The resulting urokinase has about 14 polymer  
chains per protein molecule.

Example 8) Preparation of PEG-urokinase in conditions of protection of the active site and of the surroundings thereof

20 3 mg of urokinase in 3 ml of 0.2 M borate buffer,  
pH 8.0 are added with 1.5 ml of benzamidine-Sepharose  
resin, previously washed with 30 ml of 0.5 M NaCl and  
equilibrated with the same borate buffer. The obtained  
suspension is added with 72 mg of activated PEG at a  
25 protein amino groups to PEG 1:10 molar ratio. After 60  
minutes the resin is washed 3 times with 5 ml of 0.2 M  
borate buffer, pH 8.0 and then the obtained adduct is  
removed from the resin by washing with 50 ml of 10 mM  
HCl and purified as in example 3. The obtained adduct  
30 has 13 PEG chains covalently linked per enzyme  
molecule.

Example 9) Urokinase and PEG-urokinase enzymatic activities toward a low molecular weight substrate

This activity is spectrophotometrically evaluated using carbobenzoxy L-Lys-p-nitrophenyl ester hydrochloride (Z-Lys-OpNO<sub>2</sub>) as the substrate. 65 µl of substrate (2.5 mg/ml of water) are added to 935 µl of 0.1 M K<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub> buffer, pH 6.8 containing 0 to 5 µg of urokinase. The increase in the optical density at 360 nm per minute is reported against the enzyme amounts. The esterase activity of the adducts is expressed as a percentage compared with that of the native enzyme and it is reported in Table 2.

Example 10) Urokinase and PEG-urokinase fibrinolytic activity inside a synthetic cloth

500µl of a fibrinogen solution (30 µg/ml) in 0.1 M Na<sub>2</sub>HPO<sub>4</sub>/K<sub>2</sub>HPO<sub>4</sub>, 0.5% of BSA, pH 7.2, containing 25 to 100 urokinase enzyme Units and finally 100µl of thrombin (500 UI/ml) in 0.1 M KH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>HPO<sub>4</sub> buffer pH 7.2 containing 1 mg of BSA are subsequently placed into a glass test tube (9 mm x 100 mm). The test tube is turned upside down 2 times and placed into a thermostated bath at 37°C. After 1 hour, a glass bead of 0.3 g weight is placed onto the formed cloth and the time necessary for the bead to reach the test tube bottom is evaluated. The fall time of the bead is reported on a logarithm scale against the corresponding enzyme Units. The fibrinolytic activity, expressed as the percentage compared with standard urokinase of known activity, is reported in Table 2.

Example 11) Urokinase and PEG-urokinase affinity to a synthetic cloth

200  $\mu$ l of a fibrinogen solution (4.2 mg/ml) in 0.05 M Tris HCl, 0.15 M NaCl and 0.01 M  $\text{CaCl}_2$  buffer pH 8.0 and 20  $\mu$ l of a thrombin solution (500 UI/ml) in 0.1 M  $\text{Na}_2\text{HPO}_4$  /  $\text{KH}_2\text{PO}_4$  buffer, 1% BSA%, pH 7.2, are placed into a test tube. The tubes are centrifuged at 2500 rpm for 20 minutes, to squeeze the cloth that is subsequently extruded, washed with 5 ml of 1.5 M NaCl and dried. The cloth is placed into a cuvette containing urokinase amounts varying from 0.1 to 10  $\mu$ g 0.05 M Tris HCl, 0.015 M NaCl buffer pH 8.0. Optical Density values, recorded at 3 minutes intervals, are plotted against the time<sup>2</sup>. The ratio of the concentration of the used enzyme to the obtained slopes is plotted against the urokinase concentration to obtain lines from which the affinity value of the enzyme to the cloth can be evaluated [G.A. Homandberg and T.Wai, Thrombosis Res., 55 493, 1989]. The results are reported in Table 2.

Example 12) Urokinase and PEG-urokinase activity toward plasminogen in solution

In a cuvette containing 900  $\mu$ l of 0.05 M Tris HCl buffer, 0.15 M NaCl pH 7.0, 50  $\mu$ l of a solution of 4.41 mg/ml in the same buffer Val-Leu-Lys-pNO<sub>2</sub> anilide, 40  $\mu$ l of a plasminogen 0.125-12.5 mg/ml solution and 10  $\mu$ l of an urokinase solution or a modified urokinase solution in 0.05 M Tris HCl buffer, 0.15 M NaCl, bovine serum albumin 5 mg/ml pH 7.0. The change in the optical density recorded at 405 nm is plotted against the time<sup>2</sup> expressed in minutes, to obtain the plasmin formation

rate [V. Ellis et al., J. B. C., 262 14998,1987]. In Table 2 the enzymatic activities of the various samples are reported.

**TABLE 1**  
Native trypsin and PEG-modified trypsin characteristics

	Esterolytic activity versus TAME (1) %	Protease activity versus:	
		casein (2a) %	BSA (2b) %
Native trypsin A)	100	100	100
PEG-trypsin modified randomly or with an inhibitor in solution B)	120	30	0
PEG-trypsin modified by site-protection in heterogeneous phase C)	115	100	55

(1) Values obtained in Example 4, (2) Values obtained in Example 5.



**TABLE 2**  
Native urokinase (UK) and PEG-modified UK characteristics

	Activity versus Z-Lys- $\phi$ pNO <sub>2</sub>	Fibrinolytic activity with UK inside the synthetic thrombus (2) %	Affinity to the synthetic thrombus (3) nM	Activity toward plasminogen (4) Kcat sec <sup>-1</sup>
Native UK A)	100	100	2.1	25.5
PEG-UK modified randomly or with an inhibitor in solution B)	115	0	1079	0.5
PEG-UK modified by site-protection in heterogeneous phase C)	115	21	24.3	2.7

(1) Values obtained in Example 9, (2) Values obtained in Example 10.

(3) Values obtained in Example 11, (4) Values obtained in Example 12.

CLAIMS

1. A method for the preparation of proteolytic enzymes-PEG adducts, characterized in that the proteolytic enzyme which is reacted with PEG is linked to a macromolecularized inhibitor thereof.
2. A method according to claim 1, characterized in that the proteolytic enzyme is trypsin.
3. A method according to claim 1, characterized in that the proteolytic enzyme is urokinase.
4. A method according to claim 1, characterized in that the proteolytic enzyme is selected from tissue plasminogen activator (tPA), plasmin, chymotrypsin, elastase, kallikrein.
5. A method according to claim 1, characterized in that the proteolytic enzyme inhibitor is benzamidine.
6. A method according to claim 1, characterized in that the proteolytic enzyme inhibitor is macromolecularized by grafting to a highly hydrated insoluble polysaccharide.
7. A method according to claim 6, characterized in that highly hydrated insoluble polysaccharide is Sepharose.
8. A method according to claim 1, characterized in that the heterogeneous phase consists of an aqueous suspension buffered with borate buffer to pH around 8.
9. A method according to the above claims, characterized in that a molar excess is used of PEG that is linked by means of a carbamic bond to the alpha-amino group of nor-leucine that is activated at the carboxy group as the succinimidyl ester.

10. Proteolytic enzyme - PEG adducts having proteolytic activity toward macromolecular substrates.

11. Adducts according to claim 10, which can be obtained according to the method disclosed in claims 1-

5 9.

12. The use of the adducts according to claims 10-11 as biocatalysts.

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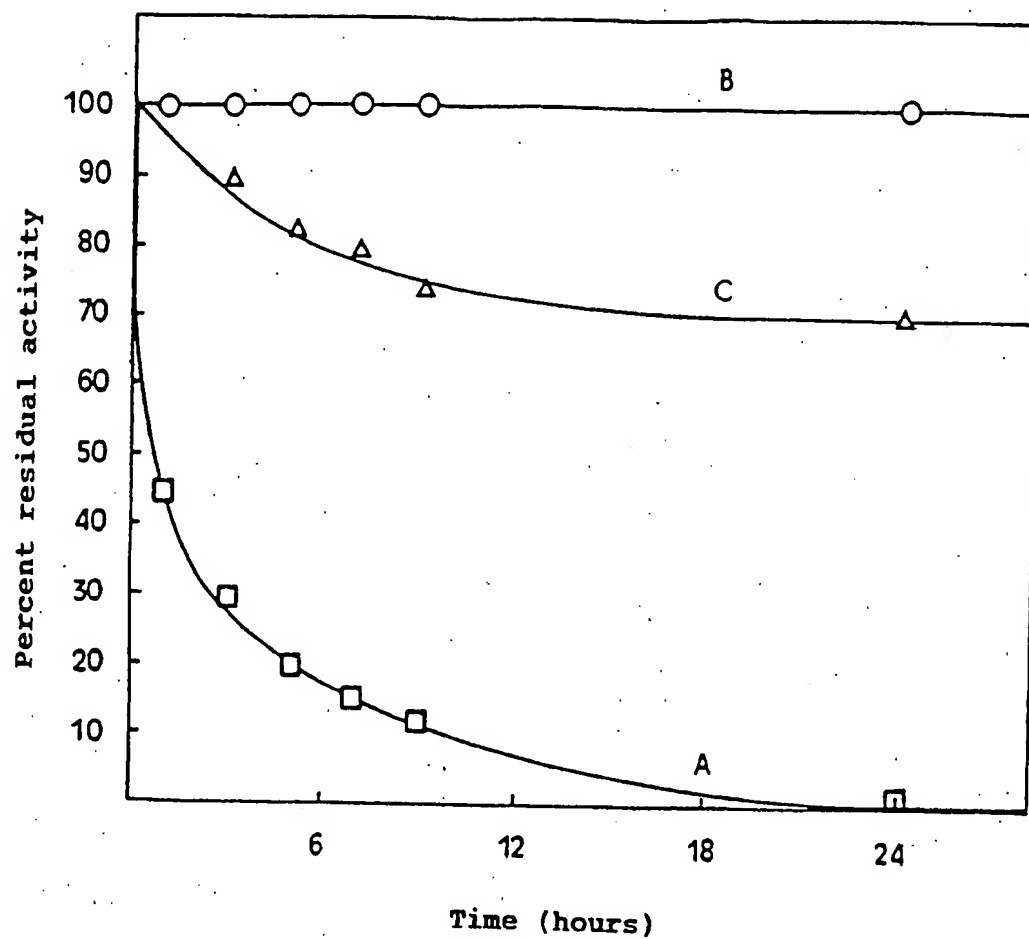


Figure 1: Autolysis of native trypsin ( $\square$ ), A; PEG-trypsin randomly modified with no site-protection (O), B; PEG-trypsin modified in heterogeneous phase in the presence of benzamidine-Sepharose ( $\Delta$ ), C.

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 93/00205

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 C12N9/96; C12N9/72; C12N9/76		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
Int.Cl. 5	C12N	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>		
Category <sup>10</sup>	Citation of Document, <sup>11</sup> with Indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	FR,A,2 515 684 (NIPPON CHEMIPHAR CO) 6 May 1983	10-12
A	--- TRENDS IN BIOTECHNOLOGY. vol. 6, no. 6, June 1988, CAMBRIDGE GB pages 131 - 134 Y. INADA ET AL 'Application of PEG-enzyme and magnetite-PEG-enzyme conjugates for biotechnological processes' see page 132	
P,X	--- JOURNAL OF BIOACTIVE AND COMPATIBLE POLYMERS vol. 8, January 1993, pages 41 - 50 P. CALICETI ET AL 'Active site protection of proteolytic enzymes by poly(ethylene glycol) surface modification' see the whole document -----	1-12
<sup>10</sup> Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "&" document member of the same patent family		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
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International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	VAN DER SCHAAL C.A.	

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		CA-A- 1203764	29-04-86
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		US-A,B 4495285	22-01-85
		US-A- 4640835	03-02-87
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